Drug-paired contextual stimuli increase dendritic spine dynamics in select nucleus accumbens neurons

Article (Accepted Version)

Drug-paired contextual stimuli increase dendritic spine dynamics in select nucleus accumbens neurons

Running Title: Conditioning & ensemble-specific spine changes

Bryan F. Singer[1*], Nancy Bubula[2], Dongdong Li[2], Magdalena M. Przybycien-Szymanska[2], Vytautas P. Bindokas[3], Paul Vezina[1,2]

[1]Committee on Neurobiology, [2]Department of Psychiatry and Behavioral Neuroscience, and [3]Department of Neurobiology, Pharmacology and Physiology, The University of Chicago, Chicago, IL, USA

Abstract: 250
Main Text: 4,072
Introduction: 739
Methods: 632
Figures: 4
Tables: 0
References: 47
Supplemental Information: 10

CORRESPONDENCE:

* Dr. Bryan F. Singer
Committee on Neurobiology
The University of Chicago
5841 S. Maryland Ave
MC 3077
Chicago, IL 60637
TEL: 01-773/702-2890
FAX: 01-773/702-0857
E-mail: bfsinger@uchicago.edu

Abstract

Repeated exposure to amphetamine leads to both associative conditioning and non-associative sensitization. Here we assessed the contribution of neuronal ensembles in the nucleus accumbens (NAcc) to these behaviors. Animals exposed to amphetamine IP or in the ventral tegmental area (VTA) showed a sensitized locomotor response when challenged with amphetamine weeks later. Both exposure routes also increased ΔFosB levels in the NAcc. Further characterization of these ΔFosB+ neurons, however, revealed that amphetamine had no effect on dendritic spine density or size, indicating that these neurons do not undergo changes in dendritic spine morphology that accompany the expression of non-associative sensitization. Additional experiments determined how neurons in the NAcc contribute to the expression of associative conditioning. A discrimination-learning procedure was used to expose rats to IP or VTA amphetamine either Paired or Unpaired with an open field. As expected, compared to Controls, Paired rats administered IP amphetamine subsequently showed a conditioned locomotor response when challenged with saline in the open field, an effect accompanied by an increase in c-Fos+ neurons in the medial NAcc. Further characterization of these c-Fos+ cells revealed that Paired rats showed an increase in the density of dendritic spines and the frequency of medium-sized spines in the NAcc. In contrast, Paired rats previously exposed to VTA amphetamine showed neither conditioned locomotion nor conditioned c-Fos+ expression. Together, these results suggest a role for c-Fos+ neurons in the medial NAcc and rapid changes in the morphology of their dendritic spines in the expression of conditioning evoked by amphetamine-paired contextual stimuli.

Introduction

Drugs of abuse, such as amphetamine, are typically administered within a complex environment containing a large number of diverse stimuli. With repeated systemic drug administration, cues come to predict the availability of the reward. This leads to the development of conditioned drug-seeking behavior in the presence of cues, thereby increasing chances of reinforcement. Interestingly, the development of associative conditioning parallels the formation of non-associative sensitized behavioral and neurochemical responding to amphetamine (Singer *et al*, 2009, 2014a, 2014b; Vezina and Leyton, 2009) and both have been implicated in escalated drug-use in humans and animal models (Leyton and Vezina, 2013, 2014).

It is possible to identify mesocorticolimbic neuroadaptations resulting from sensitization, in the absence of conditioning, by administering repeated amphetamine directly into the ventral tegmental area (VTA), the site where amphetamine is known to act to initiate the development of sensitization (Vezina, 2004). While both systemic and intra-VTA injection routes lead to the development of non-associative sensitization to amphetamine (e.g., enhanced locomotion and self-administration of drug; Vezina *et al*, 2002), only systemic amphetamine exposure leads to the generation of conditioning (Singer *et al*, 2009; Vezina and Stewart, 1990). The present experiments compared sensitization- and conditioning-related plasticity in the nucleus accumbens (NAcc) following either repeated VTA or systemic injections of amphetamine.

Cell bodies in the VTA project to and release dopamine (DA) onto medium spiny neurons (MSNs) in the NAcc. Repeated administration of DA-releasing psychostimulant drugs produces both transient and long-lasting changes in the size and density of dendritic spines on these GABAergic neurons (Dumitriu *et al*, 2012; Robinson and Kolb, 1997, 1999; Shen *et al*, 2009). These dendritic spines may be locations of memory storage in the brain (Lamprecht and LeDoux, 2004); various behavioral experiences have been shown to increase spine formation in vivo (Holtmaat *et al*, 2005, 2006; Lai *et al*, 2012; Moczulska *et al*, 2013; Muñoz-Cuevas *et al*, 2013). Importantly, by comparing the effects of intra-VTA and systemic injections of amphetamine, we recently found that spine plasticity maps onto the accrual of associative drug conditioning rather than the induction of non-associative drug sensitization (Singer *et al*, 2009). Enhanced plasticity

of dendritic spines in the NAcc core has also been documented during the retrieval of drug-related memories (Gipson *et al*, 2013). Together, these results suggest a role for dendritic plasticity in the NAcc in the expression of conditioned behaviors.

While brain-region specific changes in dendritic architecture following repeated systemic amphetamine have been reported (Robinson and Kolb, 2004; Singer *et al*, 2009), not every cell within a prescribed anatomical region likely contributes to the processing of drug-related information. In the present experiments, we characterized the dendritic morphology of neurons that contribute to the expression of either sensitization or conditioning. To accomplish this, we first visualized the dendritic profiles of NAcc neurons that accumulated the transcription factor ΔFosB following repeated drug administration (Hope *et al*, 1994; McClung *et al*, 2004). These neurons show a long-lasting increase in ΔFosB expression, even without re-exposure to drugs or cues, and have been shown to mediate enhanced locomotor responding to and self-administration of psychostimulant drugs (Colby *et al*, 2003; Kelz *et al*, 1999). In contrast to ΔFosB, the immediate early gene *c-fos* is expressed in the NAcc upon re-exposure to a drug-paired stimulus (Cruz *et al*, 2014a). Ensembles of activated c-Fos+ neurons are believed to encode behaviorally relevant conditioning information (Cruz *et al*, 2014b). Indeed, we show here that rats that do not develop conditioned responding also do not show cue-elicited c-Fos expression.

The discriminative-learning procedure we used promotes the formation of context-specific memories, along with localized activation of the NAcc shell (and not the NAcc core). Similar to other recent reports (Bossert *et al*, 2007, 2013; Everitt and Robbins, 2005; Singer *et al*, 2014a, 2014b), these experiments demonstrate a role for the NAcc shell in the processing of contextual as opposed to discrete cues. Thus, by focusing on the activation of MSNs in the NAcc shell, we highlight the existence of rapid changes in the dendritic morphology of neuronal ensembles upon retrieval of conditioned contextual memories. Given the ubiquity of drug-related stimuli in a contextual stimulus context previously associated with a drug, conditioned increases in locomotion reflect increased approach and interaction with these stimuli (Vezina and Stewart, 1987) that can promote reinstatement of drug seeking (Cruz *et al*, 2014a). These effects are consistent with the processing by neurons in the NAcc shell of the

incentive motivational properties of psychomotor stimulant drugs (Everitt and Robbins, 2005).

Methods and Materials

Subjects & Surgery

Male Sprague-Dawley rats (Harlan Sprague-Dawley; Madison, WI) weighing 250-275g on arrival were used (Supplement 1). A subset of rats (n=43) underwent stereotaxic surgery for implantation of bilateral guide cannulae aimed at the VTA, according to procedures previously described (Singer *et al*, 2009) and outlined in Supplement 1.

Behavioral Procedures

Rats were exposed to repeated amphetamine or saline injections in both locomotor sensitization (Supplement 3) and discrimination learning (Supplement 4) experiments. For sensitization studies, amphetamine (VTA, 2.5 µg per 0.5 µl/side; IP, 1.5 mg/kg) or 0.9% saline (VTA, 0.5 µl/side; IP, 1.0 ml/kg) were administered in rats' home cages once every 3 days for a total of 4 VTA microinjections or 5 IP injections. While these same doses were used for the conditioning studies, injections were given in specific contexts (Figure S1). Briefly, Paired rats received amphetamine in an open field and saline in their home cages. In contrast, Unpaired rats received saline in the open field and amphetamine in the home cage. Control rats received saline injections in both environments. For all studies, locomotion was recorded in the open field chambers (Supplement 2).

Immunoblotting

Separate rats were exposed to amphetamine or saline (IP, AMPH n=9, SAL n=11; VTA, AMPH n=8, SAL n=5; locomotor data were not collected; Supplement 3) and sacrificed via rapid decapitation 1-week later. Brains were immediately harvested and then frozen for later analysis of ΔFosB expression in the NAcc (Figure 1; Supplement 5).

Immunohistochemistry & Cell Counting

Rats were fully anesthetized [ketamine (100 mg/kg, IP) and xylazine (10 mg/kg, IP)] and perfused intracardiacally with phosphate-buffered saline (1XPBS) and 1.5% paraformaldehyde. Brains were then harvested (Supplement 6) and 100 µm coronal sections were obtained. NAcc cell counting and DiI injections were performed in slices ~2.10±0.20 mm from bregma (Paxinos and Watson, 1998). Free-floating sections were stained for NeuN, either c-Fos or ΔFosB expression or both (cell counting). For DiI injections, free-floating sections were stained for either ΔFosB or c-Fos expression.

DiI Injections & Dendritic Morphology

DiI injections were performed in immunostained tissue as described (Supplements 6, 7; Figure S3; 120 cells and 20,349 spines analyzed). For rats exposed to repeated systemic amphetamine or saline in the sensitization experiment, ΔFosB+ or unlabeled neurons (ΔFosB-) were identified and injected with DiI (Figure 2; n=3-4 rats/group). For the discrimination learning experiment (Figure 4; n=4 rats/group), c-Fos+ or unlabeled (c-Fos-) neurons were injected with DiI. For all cell injections (3.24±0.19 cell-type/rat), a pipette was positioned onto the plasma membrane and DiI was deposited via an injection of positive current. Second-order dendrites from DiI-injected neurons were imaged using a confocal microscope. Dendrites underwent automatic software reconstruction (Imaris, Bitplane; Supplement 7; Shen *et al*, 2008, 2009). Dendritic spine tip densities were higher than overall spine densities because some protrusions had multiple heads, a phenomenon associated with conditioning (Geinisman *et al*, 2001).

Data Analysis

Sensitized locomotion, ΔFosB expression, and overall spine (tip) densities for ΔFosB+/- neurons were analyzed using independent t-tests. Tests of conditioned locomotion, c-Fos, and ΔFosB expression were analyzed using one-way ANOVAs with conditioning group as the between factor. Changes in overall dendritic spine (tip) density after the conditioning test were also analyzed using one-way ANOVAs [separately for c-Fos+ and c-Fos- neurons]. Finally, spine tip diameters (for both ΔFosB

and c-Fos studies) were measured across neurons and placed into bins to permit analytical comparisons to previously described results (Dumitriu *et al*, 2012; Shen *et al*, 2009). Two-way between-within ANOVAs were performed to determine the presence of conditioning group X tip diameter interactions. A significant interaction allowed for analysis of tip size frequency in specific pre-defined diameter bins using post-hoc Scheffé tests (Kirk, 1968). Comparing frequencies of specific tip diameters to the overall tip density helped differentiate between de novo spine formation and shifts in the distribution of existing spines sizes.

Results

Non-Associative Sensitization

Repeated intermittent exposure to amphetamine 1) enhances the ability of the drug to elicit psychomotor behavior (sensitization), and 2) increases expression of the transcription factor ΔFosB in the NAcc. We confirmed that these long-lasting adaptations result from either systemic (IP) or intra-VTA (microinjected) amphetamine exposure. On tests conducted one-week following drug exposure, both routes of drug administration resulted in sensitized locomotor responding to a systemic amphetamine challenge relative to rats that previously received saline (Figure 1A-B; IP, $t_{10}=4.52$, $p<0.001$; VTA, $t_{11}=3.75$, $p<0.01$). Similarly, we found in a separate group of rats that exposure to either IP (Figure 1C; $t_{18}=2.14$, $p<0.05$) or VTA (Figure 1D; $t_{11}=2.25$, $p<0.05$) amphetamine increased ΔFosB expression in the NAcc relative to saline exposed controls. Together, these findings demonstrate that either repeated systemic or intra-VTA amphetamine exposure is sufficient to induce behavioral sensitization and long-lasting ΔFosB expression. It is possible that elevated ΔFosB levels facilitate gene transcription needed to support the expression of sensitization (Colby *et al*, 2003; Hu *et al*, 2002; Kelz *et al*, 1999).

Despite these findings, VTA-amphetamine does not increase dendritic spine density in the NAcc (Singer *et al*, 2009). Since both routes of drug administration result in behavioral sensitization, the observed dissociation suggests that ΔFosB-expression is not sufficient to increase the dendritic spine plasticity associated with repeated IP amphetamine (Robinson and Kolb, 2004; Singer *et al*, 2009). Therefore, we assessed

the dendritic spine morphology and density of ΔFosB+ and ΔFosB- neurons in the NAcc shell, one-week after exposure to repeated IP amphetamine or saline. To accomplish this, we immunohistochemically identified ΔFosB+ neurons and specifically targeted these cells for injection with the fluorescent neuronal tracer DiI. We then imaged these neurons using confocal microscopy to create three-dimensional reconstructions of their neuroanatomy (Figure 2D-F).

In ΔFosB+ neurons, no significant differences were detected in the sizes of dendritic spine heads (tip-diameter) between rats previously exposed to amphetamine or saline [Figure 2A; Repeated measures ANOVA; between group factor (amphetamine vs. saline), $F_{1,5}$=0.23, ns; within factor (diameter), $F_{5,25}$=143.36, p<0.001; interaction (diameter x group), $F_{5,25}$=0.44, ns]. Similarly, ΔFosB+ neurons sampled from amphetamine- and saline-exposed rats did not differ in their overall spine density (Figure 2C top; independent t-test; t_5=0.80, ns) or spine tip density (Figure 2C bottom; independent t-test; t_5=0.92, ns). As a control, we also analyzed the spine characteristics of ΔFosB- neurons in the NAcc. Again, the distribution of spine tip diameters was not different between rats that received amphetamine or saline one-week earlier [Figure 2B; Repeated measures ANOVA; between group factor (amphetamine vs. saline), $F_{1,4}$=1.88, ns; within factor (diameter), $F_{5,20}$=171.13, p<0.001; interaction (diameter x group), $F_{5,20}$=1.51, ns]. Overall dendritic spine density (Figure 2C top; independent t-test; t_4=1.13, ns) and spine tip density (Figure 2C bottom; independent t-test; t_4=0.98, ns) also did not differ between the two groups.

We draw several conclusions from these results. First, high-resolution imaging and reconstruction techniques revealed that long-lasting changes in dendritic spine properties are not present after cessation from repeated sensitizing psychostimulant exposure regimens. Instead, as indicated below, we hypothesize that repeated drug exposure allows for long-term plasticity that facilitates rapid changes in dendritic spines specifically following re-exposure to conditioned stimuli predictive of the drug. Second, we hypothesize that changes in dendritic spines are not observed in ΔFosB+ neurons because these cells mediate non-associative sensitization rather than associative conditioning. Indeed, VTA-amphetamine exposed rats show enhanced behavioral responding to amphetamine along with increased ΔFosB levels and do so in the

absence of conditioned behavioral responding to drug-paired stimuli (Figure 3B; Singer *et al* 2009).

Associative Conditioning

Since neurons essential for the expression of behavioral sensitization (ΔFosB+) do not show long-lasting changes in dendritic spines (Figure 2), we next determined whether neurons participating in the expression of conditioned behavioral responses undergo dendritic alterations. To identify these neurons, rats underwent a discriminative learning procedure in which an open field was associated with the drug for some rats (Paired) and not associated with the drug for others (Unpaired). Unpaired rats received amphetamine elsewhere (Figure S1). Control rats received saline injections in both environments. This drug-conditioning regimen was conducted with IP or intra-VTA injections in separate groups of rats. Similar to what we have previously reported (Singer *et al*, 2009; Vezina and Stewart, 1990), when tested one-week later, only Paired rats displayed a conditioned locomotor response after being reintroduced to the open field following a saline injection (Figure 3A; One-way ANOVA, 30-minute total locomotion; $F_{2,12}=7.41$, $p<0.01$). Post-hoc Scheffé tests demonstrated that Paired rats showed significantly enhanced conditioned locomotion relative to the Unpaired and Control rats ($p<0.05$) which did not differ from one another. In contrast, previous VTA amphetamine administration did not support the development of associative conditioning in Paired rats (Figure 3B; One-way ANOVA, 30-minute total ambulation; $F_{2,14}=0.92$, ns). Altogether, these results indicate that drug-environment associations are formed following systemic, but not VTA, amphetamine exposure.

Based on these findings, we predicted that re-exposure to the drug-paired open field would elicit conditioned cellular activation only in Paired rats demonstrating conditioned behavioral responding. We thus began by using immunohistochemistry to assess changes in expression of the immediate early gene product c-Fos following reintroduction to the drug-paired context and expression of conditioned locomotion. As expected, rats that showed conditioned behavioral responding (Paired, IP AMPH) also showed conditioned increases in c-Fos expression in the NAcc shell [Figure 3C; One-way ANOVA, c-Fos/NeuN, % Control; $F_{2,10}=5.18$, $p<0.05$; Scheffé post-hocs, Paired

(264.25±30.88 cells/rat) vs. Unpaired (180.60±16.17 cells/rat) or Control (216.50±25.91 cells/rat), $p<0.05$]. This effect was not expected nor was it observed in the NAcc core (data not shown; one-way ANOVA, c-Fos/NeuN, $F_{2,23}=0.57$, ns) as contextual stimuli are processed in the NAcc shell and not the NAcc core (Cruz *et al*, 2014a; Singer *et al*, 2014a, 2014b). Importantly, Paired rats that previously received intra-VTA amphetamine displayed neither conditioned locomotion nor increases in c-Fos expression in the NAcc shell [Figure 3D; One-way ANOVA, c-Fos/NeuN, % Control; $F_{2,14}=0.18$, ns; Paired (590.33±58.72 cells/rat), Unpaired (676.00±62.73 cells/rat), Control (575.50±44.76 cells/rat)]. Thus, comparison of the results suggests that conditioned neuronal activation in the NAcc shell and behavioral responding to contextual stimuli are related.

In contrast, assessment of ΔFosB in rats subjected to the IP injection conditioning procedure showed that, despite the selective expression of locomotor conditioning in Paired rats (Figure 3A), ΔFosB expression in the NAcc shell was elevated in both Paired and Unpaired rats relative to Controls [Figure 3E; One-way ANOVA, ΔFosB/NeuN, % Control; $F_{2,23}=5.49$, $p<0.05$; Scheffé post-hoc, Paired (452.06±30.78 cells/rat) and Unpaired (429.17±17.73 cells/rat) vs. Control (340.30±26.55 cells/rat), $p<0.05$]. A small number of c-Fos+ neurons were also found to be ΔFosB+ after re-exposure to the amphetamine-paired environment (<10%; 40.73±5.82 cells/rat; 3-dimensional confocal analysis). This number is far lower than that observed in a previous report (>60%; Mattson *et al*, 2008) and >10-fold lower than the number of c-Fos+ neurons observed in the present experiments, indicating that context-dependent expression of c-Fos itself reflects activity in an ensemble of neurons that is critical for the expression of conditioned responding to contextual stimuli.

It has been documented that amphetamine conditioning is associated with increases in dendritic spine density in the NAcc (Singer *et al*, 2009). Since c-Fos+ cells may be essential to the expression of conditioned responding (Figure 3), we investigated whether these neurons undergo dendritic changes upon re-exposure to the drug-paired context. One-week after the induction of conditioning (IP injections), rats were re-exposed to the open field for 30-minutes, sacrificed, and their brains rapidly removed for subsequent c-Fos immunohistochemistry and DiI injections. Dendritic reconstructions revealed strikingly different results from those observed in ΔFosB+

neurons. Paired rats showed a significantly different distribution of dendritic spine head sizes relative to both Unpaired and Control rats [Figure 4A; Repeated measures ANOVA; between group factor (Paired, Unpaired, Control), $F_{2,9}$=3.45, p<0.05; within factor (diameter), $F_{5,45}$=285.48, p<0.001; interaction (diameter x group), $F_{10,45}$=7.39, p<0.001]. Closer examination revealed that Paired rats showed a significant increase in the frequency of medium-sized spine tips (0.45-0.60µm diameters) compared to Unpaired and Control rats (Post-hoc Scheffé tests, p<0.001). In contrast, no group differences were observed in spine tip diameters in c-Fos- neurons [Figure 4B; Repeated measures ANOVA; between group factor (Paired, Unpaired, Control), $F_{2,9}$=0.35, ns; within factor (diameter), $F_{5,45}$=218.45, p<0.001; interaction (diameter x group), $F_{10,45}$=0.88, ns]. These findings obtained in NAcc shell MSNs are consistent with adaptations processing information necessary for contextual conditioning and do not argue against similar changes occurring in the NAcc core. Indeed, both changes in c-Fos expression (Kufahl *et al*, 2009) and in spine morphology (Gipson *et al*, 2013) have been reported in the NAcc core following the presentation of discrete drug-paired cues.

While changes in spine tip diameter are suggestive of rapid plasticity, these could reflect either the altered morphology of existing spines or the *de novo* formation of new spines. We, therefore, investigated whether re-exposure to the open field resulted in altered overall spine density and tip density in c-Fos+ and c-Fos- neurons (Figure 4C). Relative to Unpaired and Control groups, c-Fos+ neurons sampled from Paired rats displayed conditioned increases in both dendritic spine density (One-way ANOVA, spines/µm; $F_{2,9}$=6.33, p<0.05; Post-hoc Paired vs. Unpaired & Control, p<0.05) and tip density (One-way ANOVA, tips/µm; $F_{2,9}$=4.67, p<0.05; Post-hoc Paired vs. Unpaired & Control, p<0.05). Finally, neither spine density (One-way ANOVA, spines/µm; $F_{2,9}$=0.07, ns) nor spine tip density (One-way ANOVA, spine tips/µm; $F_{2,9}$=0.51, ns) modifications were observed in c-Fos- neurons. Together, the selective increase in both spine and tip density in c-Fos+ neurons from Paired rats suggests that these are newly-formed spines responding to conditioned stimuli. This effect was clearly not due to simple exposure to amphetamine as it was absent in Unpaired rats that received the same number of amphetamine injections as Paired rats during conditioning.

Discussion

Repeated intermittent exposure to drugs of abuse, a pattern often associated with initial casual experimentation, can lead to long-lasting neuroadaptations capable of regulating drug-induced behaviors and escalating drug-use in humans and animal models (Leyton and Vezina, 2013, 2014). Intermittent exposure, especially to psychostimulants like amphetamine and cocaine, is known to produce sensitization (Vezina, 2004), but as drugs are typically administered in the presence of a vast number of disparate environmental stimuli, the formation of multiple conditioned drug-stimulus associations is also ensured (Vezina and Leyton, 2009). Therefore, a major challenge for characterizing the neuroadaptations underlying associative conditioning is to study this form of plasticity in isolation. By comparing the effects of repeated systemic and intracranial VTA injections of amphetamine in a discrimination-learning procedure, we were able to isolate neuroadaptations specific to amphetamine-induced associative conditioning.

Here we show that re-exposure to a context previously associated with systemic amphetamine increases the number of c-Fos+ neurons in the medial NAcc shell (Figure 3) and increases spine density and the frequency of medium sized spine tip diameters (Figure 4) selectively in these cells. As spine number and volume are proportional to the area of the postsynaptic density and to the synaptic content of AMPA receptors (Holtmaat and Svoboda, 2009), these adaptations observed in NAcc shell c-Fos+ MSNs are well positioned to enable heightened conditioned responding to a drug-paired context. Similar c-Fos associated recruitment of AMPA receptors to mushroom-type spines was reported after fear conditioning (Matsuo *et al*, 2008).

As with repeated systemic amphetamine injections, repeated exposure to amphetamine in the VTA leads to enhanced ΔFosB expression in the NAcc and behavioral sensitization (Figure 1). However, unlike the former, VTA microinjections of the drug do not lead to associative memory formation as detected by the lack of conditioned locomotor responding to previously drug-paired stimuli and the failure of these stimuli to increase c-Fos expression in the NAcc (Figure 3). In addition, the number of ΔFosB+ neurons is increased in both Paired and Unpaired rats regardless of the environment in which they were exposed to systemic amphetamine (Figure 3E) and,

unlike c-Fos+ neurons, they do not exhibit changes in dendritic morphology (Figure 2) even though a large number of drug-associated stimuli (e.g., odor, tactile, visual experimenter and procedural cues) were present in the period leading to tissue harvest. Notably, unlike c-Fos, ΔFosB levels are slow to accumulate (Hope *et al*, 1994) and do not match the fast time-course of conditioned locomotion, making it difficult for the two to be associated. These findings are consistent with a role for c-Fos expression in associative learning (Cruz *et al*, 2014a; Matsuo *et al*, 2008; Ostrander *et al*, 2003) and further support a strong relationship between c-Fos associated changes in dendritic morphology in the NAcc and the expression of conditioning. These effects are not observed in c-Fos- neurons that do not participate in the expression of conditioning. Indeed, as IP but not VTA amphetamine leads to conditioning, the observed changes in spine morphology likely reflect associative memories manifesting as the stimulus-induced recruitment of newly-formed spines favoring medium-sized tip diameters. In contrast, the increase in ΔFosB observed here and by others (Colby *et al*, 2003; Kelz *et al*, 1999) likely reflects the encoding of information mediating non-associative sensitization.

Importantly, manipulations, such as inhibiting cyclin-dependent kinase 5 (cdk5), that are known to prevent drug-induced spine proliferation in the NAcc (Norrholm *et al*, 2003) also prevent the development of conditioning but spare sensitization (Singer *et al*, 2014b). While we did not observe spine alterations in ΔFosB+ neurons in the present study, it remains possible under some conditions for changes to occur in these neurons that enable the development of conditioned associations, as ΔFosB is a transcription factor for the *cdk5* gene. For example, morphological changes may occur in some ΔFosB+ neurons co-expressing c-Fos or at a time-point different from that examined in the present experiments. Indeed, genetic overexpression of ΔFosB does increase spine density and the existence of immature spines in the NAcc shortly after a cocaine injection (Grueter *et al*, 2013; Maze *et al*, 2010). However, the viral-mediated nature of these changes, potentially elevating ΔFosB expression above endogenous levels and in neurons not participating in drug-cue-encoding ensembles, make these findings difficult to interpret in light of the present results. The goal of the present experiments was to assess the ability of drug-paired stimuli to induce morphological changes in the dendritic

spines of select neurons in the NAcc. Clearly, future experiments will need to characterize further the time course of these effects and the sequence of signaling events underlying them. Our current findings support a strong relationship between rapid cue-induced changes in the dendritic spine morphology of c-Fos+ neurons in the NAcc and the expression of conditioned responding to drug-paired stimuli.

The selective increase in the frequency of spines with medium tip diameters (0.45-0.60μm) in c-Fos+ neurons in the NAcc shell of Paired rats is similar to changes observed in this dendritic spine size in randomly-selected neurons in the NAcc core following cocaine or exposure to discrete cues (Dumitriu *et al*, 2012; Gipson *et al*, 2013). Interestingly, Golgi-Cox staining used in previous studies is also most sensitive for detecting changes in these medium-sized spines (Shen *et al*, 2009). Together, these findings suggest the importance of this particular spine subtype in mediating behavioral responses. It is unknown why these changes in spines were restricted to medium-sized tip diameters, but it may be that these spine head sizes are volatile and susceptible to rapid changes based on increased or decreased synaptic input (Matsuo *et al*, 2008).

A common view in models of learning is that changes in the morphology of dendritic spines and the long-term stabilization of these changes embody the neural representation of persistent memories (Koleske, 2013; Lamprecht and LeDoux, 2004; Muñoz-Cuevas *et al*, 2013; Roberts *et al*, 2010; Xu *et al*, 2009). The present findings, as well as the transient effects reported elsewhere (Gipson *et al*, 2013), are not consistent with these models according to which repeated drug exposure leads to long-lasting, static changes in spine morphology. In the present experiments, both Paired and Unpaired rats were exposed to the same number of systemic amphetamine injections during conditioning, yet only Paired rats showed a conditioned response when tested in the drug-paired context. Thus, the increases in spine density and spine tip diameter observed in the NAcc of Paired rats following re-exposure to the drug-paired stimuli were rapid (~30 minutes), again, as they were not observed in Unpaired rats tested in the absence of the drug-associated stimuli. Rather than static changes in spine morphology, these findings suggest that associative conditioning requires neuroadaptations in NAcc MSNs that can allow drug-paired stimuli, when present, to

evoke rapid conditioned changes in dendritic spines capable of enabling the expression of a behavioral conditioned response.

Financial Disclosures

This research was funded by US National Institutes of Health (NIH) grants R01 DA09397 (PV), T32 DA07255 (BFS), and F31 DA030021-01A1 (BFS). Work by B.F. Singer was partially funded by the Chicago Biomedical Consortium with support from The Searle Funds at The Chicago Community Trust. There are no other conflicts of interest or financial disclosures for Bryan F. Singer, Nancy Bubula, Dongdong Li, Magdalena M. Przybycien-Szymanska, Vytautas P. Bindokas, or Paul Vezina.

Acknowledgments

We would like to thank Dr. Ronald Thisted for advice regarding the statistical analysis of changes in dendritic spine morphology. We would also like to thank Elizabeth Lee and Justin Forneris for their assistance with the ΔFosB+ and c-Fos+ cell counting.

References

Bossert JM, Marchant NJ, Calu DJ, Shaham Y (2013). The reinstatement model of drug relapse: recent neurobiological findings, emerging research topics, and translational research. *Psychopharmacology (Berl)* **229**: 453–76.

Bossert JM, Poles GC, Wihbey K a, Koya E, Shaham Y (2007). Differential effects of blockade of dopamine D1-family receptors in nucleus accumbens core or shell on reinstatement of heroin seeking induced by contextual and discrete cues. *J Neurosci* **27**: 12655–63.

Colby CR, Whisler K, Steffen C, Nestler EJ, Self DW (2003). Striatal cell type-specific overexpression of DeltaFosB enhances incentive for cocaine. *J Neurosci* **23**: 2488–93.

Cruz FC, Babin KR, Leao RM, Goldart EM, Bossert JM, Shaham Y, *et al* (2014a). Role of Nucleus Accumbens Shell Neuronal Ensembles in Context-Induced

Reinstatement of Cocaine-Seeking. *J Neurosci* **34**: 7437–7446.

Cruz FC, Javier Rubio F, Hope BT (2014b). Using c-fos to study neuronal ensembles in corticostriatal circuitry of addiction. *Brain Res* In Pressdoi:10.1016/j.brainres.2014.11.005.

Dumitriu D, Laplant Q, Grossman YS, Dias C, Janssen WG, Russo SJ, *et al* (2012). Subregional, dendritic compartment, and spine subtype specificity in cocaine regulation of dendritic spines in the nucleus accumbens. *J Neurosci* **32**: 6957–66.

Everitt BJ, Robbins TW (2005). Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. *Nat Neurosci* **8**: 1481–9.

Geinisman Y, Berry RW, Disterhoft JF, Power JM, Zee EA Van der (2001). Associative learning elicits the formation of multiple-synapse boutons. *J Neurosci* **21**: 5568–73.

Gipson CD, Kupchik YM, Shen H, Reissner KJ, Thomas CA, Kalivas PW (2013). Relapse induced by cues predicting cocaine depends on rapid, transient synaptic potentiation. *Neuron* **77**: 867–72.

Grueter BA, Robison AJ, Neve RL, Nestler EJ, Malenka RC (2013). ΔFosB differentially modulates nucleus accumbens direct and indirect pathway function. *Proc Natl Acad Sci U S A* **110**: 1923–8.

Holtmaat A, Svoboda K (2009). Experience-dependent structural synaptic plasticity in the mammalian brain. *Nat Rev Neurosci* **10**: 647–58.

Holtmaat A, Wilbrecht L, Knott GW, Welker E, Svoboda K (2006). Experience-dependent and cell-type-specific spine growth in the neocortex. *Nature* **441**: 979–83.

Holtmaat AJGD, Trachtenberg JT, Wilbrecht L, Shepherd GM, Zhang X, Knott GW, *et al* (2005). Transient and persistent dendritic spines in the neocortex in vivo. *Neuron* **45**: 279–91.

Hope BT, Nye HE, Kelz MB, Self DW, Iadarola MJ, Nakabeppu Y, *et al* (1994). Induction of a long-lasting AP-1 complex composed of altered Fos-like proteins in brain by chronic cocaine and other chronic treatments. *Neuron* **13**: 1235–44.

Hu X-T, Koeltzow TE, Cooper DC, Robertson GS, White FJ, Vezina P (2002). Repeated ventral tegmental area amphetamine administration alters dopamine D1 receptor signaling in the nucleus accumbens. *Synapse* **45**: 159–70.

Kelz MB, Chen J, Carlezon W a, Whisler K, Gilden L, Beckmann a M, *et al* (1999). Expression of the transcription factor deltaFosB in the brain controls sensitivity to cocaine. *Nature* **401**: 272–6.

Kirk R (Brooks/Cole: Pacific Grove, CA, 1968). *Experimental design: procedures for the behavioral sciences*. .

Koleske AJ (2013). Molecular mechanisms of dendrite stability. *Nat Rev Neurosci* **14**: 536–50.

Kufahl PR, Zavala AR, Singh A, Thiel KJ, Dickey ED, Joyce JN, *et al* (2009). c-Fos expression associated with reinstatement of cocaine-seeking behavior by response-contingent conditioned cues. *Synapse* **63**: 823–35.

Lai CSW, Franke TF, Gan W-B (2012). Opposite effects of fear conditioning and extinction on dendritic spine remodelling. *Nature* **483**: 87–91.

Lamprecht R, LeDoux J (2004). Structural plasticity and memory. *Nat Rev Neurosci* **5**: 45–54.

Leyton M, Vezina P (2013). Striatal ups and downs: Their roles in vulnerability to addictions in humans. *Neurosci Biobehav Rev* **37**: 1999–2014.

Leyton M, Vezina P (2014). Dopamine ups and downs in vulnerability to addictions: a neurodevelopmental model. *Trends Pharmacol Sci* **35**: 268–76.

Matsuo N, Reijmers L, Mayford M (2008). Spine-type-specific recruitment of newly synthesized AMPA receptors with learning. *Science* **319**: 1104–7.

Mattson BJ, Koya E, Simmons DE, Mitchell TB, Berkow A, Crombag HS, *et al* (2008). Context-specific sensitization of cocaine-induced locomotor activity and associated neuronal ensembles in rat nucleus accumbens. *Eur J Neurosci* **27**: 202–12.

Maze I, Covington HE, Dietz DM, LaPlant Q, Renthal W, Russo SJ, *et al* (2010). Essential Role of the Histone Methyltransferase G9a in Cocaine-Induced Plasticity.

Science (80-) **327**: 213–216.

McClung C a, Ulery PG, Perrotti LI, Zachariou V, Berton O, Nestler EJ (2004). DeltaFosB: a molecular switch for long-term adaptation in the brain. *Brain Res Mol Brain Res* **132**: 146–54.

Moczulska KE, Tinter-Thiede J, Peter M, Ushakova L, Wernle T, Bathellier B, *et al* (2013). Dynamics of dendritic spines in the mouse auditory cortex during memory formation and memory recall. *Proc Natl Acad Sci U S A* **110**: 18315–20.

Muñoz-Cuevas FJ, Athilingam J, Piscopo D, Wilbrecht L (2013). Cocaine-induced structural plasticity in frontal cortex correlates with conditioned place preference. *Nat Neurosci* **16**: 1367–9.

Norrholm SD, Bibb JA, Nestler EJ, Ouimet CC, Taylor JR, Greengard P (2003). Cocaine-induced proliferation of dendritic spines in nucleus accumbens is dependent on the activity of cyclin-dependent kinase-5. *Neuroscience* **116**: 19–22.

Ostrander MM, Badiani A, Day HEW, Norton CS, Watson SJ, Akil H, *et al* (2003). Environmental context and drug history modulate amphetamine-induced c-fos mRNA expression in the basal ganglia, central extended amygdala, and associated limbic forebrain. *Neuroscience* **120**: 551–71.

Paxinos G, Watson C (Academic Press: 1998). *The rat brain in stereotaxic coordinates*. *San Diego Acad Press* **Second Edi**: .

Roberts TF, Tschida KA, Klein ME, Mooney R (2010). Rapid spine stabilization and synaptic enhancement at the onset of behavioural learning. *Nature* **463**: 948–52.

Robinson TE, Kolb B (1997). Persistent structural modifications in nucleus accumbens and prefrontal cortex neurons produced by previous experience with amphetamine. *J Neurosci* **17**: 8491–7.

Robinson TE, Kolb B (1999). Alterations in the morphology of dendrites and dendritic spines in the nucleus accumbens and prefrontal cortex following repeated treatment with amphetamine or cocaine. *Eur J Neurosci* **11**: 1598–604.

Robinson TE, Kolb B (2004). Structural plasticity associated with exposure to drugs of abuse. *Neuropharmacology* **47 Suppl 1**: 33–46.

Shen H, Sesack SR, Toda S, Kalivas PW (2008). Automated quantification of dendritic spine density and spine head diameter in medium spiny neurons of the nucleus accumbens. *Brain Struct Funct* **213**: 149–57.

Shen H, Toda S, Moussawi K, Bouknight A, Zahm DS, Kalivas PW (2009). Altered dendritic spine plasticity in cocaine-withdrawn rats. *J Neurosci* **29**: 2876–84.

Singer BF, Forneris J, Vezina P (2014a). Inhibiting cyclin-dependent kinase 5 in the nucleus accumbens enhances the expression of amphetamine-induced locomotor conditioning. *Behav Brain Res* **275**: 96–100.

Singer BF, Neugebauer NM, Forneris J, Rodvelt KR, Li D, Bubula N, *et al* (2014b). Locomotor conditioning by amphetamine requires cyclin-dependent kinase 5 signaling in the nucleus accumbens. *Neuropharmacology* **85C**: 243–252.

Singer BF, Tanabe LM, Gorny G, Jake-Matthews C, Li Y, Kolb B, *et al* (2009). Amphetamine-induced changes in dendritic morphology in rat forebrain correspond to associative drug conditioning rather than nonassociative drug sensitization. *Biol Psychiatry* **65**: 835–40.

Vezina P (2004). Sensitization of midbrain dopamine neuron reactivity and the self-administration of psychomotor stimulant drugs. *Neurosci Biobehav Rev* **27**: 827–39.

Vezina P, Leyton M (2009). Conditioned cues and the expression of stimulant sensitization in animals and humans. *Neuropharmacology* **56 Suppl 1**: 160–8.

Vezina P, Lorrain DS, Arnold GM, Austin JD, Suto N (2002). Sensitization of midbrain dopamine neuron reactivity promotes the pursuit of amphetamine. *J Neurosci* **22**: 4654–62.

Vezina P, Stewart J (1987). Conditioned locomotion and place preference elicited by tactile cues paired exclusively with morphine in an open field. *Psychopharmacology (Berl)* **91**: 375–80.

Vezina P, Stewart J (1990). Amphetamine administered to the ventral tegmental area but not to the nucleus accumbens sensitizes rats to systemic morphine: lack of conditioned effects. *Brain Res* **516**: 99–106.

Xu T, Yu X, Perlik AJ, Tobin WF, Zweig JA, Tennant K, *et al* (2009). Rapid formation

and selective stabilization of synapses for enduring motor memories. *Nature* **462**: 915–9.

Figure Legends

Figure 1. Exposure to amphetamine IP (left) or in the VTA (right) enhances subsequent locomotor responding to amphetamine and ΔFosB expression in the NAcc.

Testing was conducted 1-week following exposure. All rats were administered an IP challenge injection of amphetamine. Rats previously exposed to amphetamine (AMPH) either IP (**A**) or in the VTA (**B**) showed a sensitized locomotor response to amphetamine compared to saline (SAL) exposed rats. Data are shown as group mean (±SEM) locomotor counts over the 2-hour test as well as group mean test total locomotor counts (+SEM) in the insets. Similarly, both IP (**C**) and VTA (**D**) exposure to amphetamine led to increased ΔFosB expression in the NAcc 1-week later. Western blot results are depicted as ΔFosB/tubulin ratios and shown as % of SAL controls. Example blots are displayed below the graphs. *****, $p<0.05$; ******, $p<0.01$; *******, $p<0.001$; amphetamine-exposed relative to saline-exposed rats. Numbers at the base of each column indicate rats/group.

Figure 2. Exposure to amphetamine IP had no effect on the characteristics of dendritic spines in ΔFosB+ or ΔFosB- neurons in the NAcc shell.

Testing was conducted 1-week following exposure. Compared to saline (SAL) exposure, no effect of amphetamine (AMPH) exposure was detected on the distribution of spine tip diameters in (**A**) ΔFosB+ and (**B**) ΔFosB- neurons. For each reconstructed dendrite, the number of spines with tip diameters falling within 0.15µm intervals (e.g.,

0.30-0.45 µm; listed as 0.45 µm on the abscissa) was counted and the frequency of spines per µm of dendrite was determined. (**C**) Exposure to amphetamine also produced no effects on spine or spine tip density in ΔFosB+ or ΔFosB- neurons. Data are shown as group means (+SEM). (**D**) DiI labeled dendritic segment following deconvolution processing. Images were taken on a Leica SP5 confocal microscope (63X oil objective with 4X digital zoom). (**E**) Reconstruction of the dendritic segment using the Imaris software filament tracer module. (**F**) Surface reconstruction of the dendritic segment used to determine the quality of the DiI staining. Numbers in parentheses indicate rats, cells/group. Measures obtained from the cells of each rat (2-6/rat) were averaged and ANOVA conducted on these values. ANOVA conducted on un-averaged cell values yielded identical results, indicating homogeneity between cells for the different group measures (see Supplement 7).

Figure 3. Exposure to amphetamine IP (left) but not in the VTA, (right) produces locomotor conditioning and an amphetamine-paired stimulus-induced increase in c-Fos+ neurons in the NAcc shell.

Testing was conducted 1-week following exposure. All rats were administered saline IP before placement in the open field. (**A**) Paired rats previously administered amphetamine IP displayed conditioned locomotion compared to Unpaired and Control rats. (**B**) This was not observed in Paired rats previously administered amphetamine into the VTA. Test results are shown as group mean (±SEM) locomotor counts over the 30-minute test as well as group mean (+SEM) test total locomotor counts in the insets. Paralleling the locomotor results, an increase in c-Fos+ neurons was observed in the NAcc shell of Paired rats previously exposed to amphetamine IP (**C**; Control, 216.50±25.91 cells/rat) but not in the VTA (**D**; Control, 575.50±44.76 cells/rat). In contrast, all rats previously administered amphetamine IP displayed elevated ΔFosB expression in the NAcc independent of context (**E**), consistent with a role for ΔFosB+ neurons in the accrual of sensitization and for c-Fos+ neurons in the expression of conditioning (Control, 340.30±26.55 cells/rat). Data are shown as group mean (+SEM)

number of neurons expressed as % of Control. (**F**) Regions sampled for cell counting in the NAcc shell. Cell counts from all 6 regions were combined for each rat. The representative images from a single plain-of-focus show staining of c-Fos, ΔFosB, and NeuN expression in the NAcc. The line drawing is from Paxinos and Watson (1998) and represents the caudal surface of a 100 μm thick section. Number to the left indicates mm from bregma. *****, $p<0.05$; compared to Unpaired and Control (**A** and **C**). †, $p<0.05$; compared to Paired and Unpaired (**E**). Numbers at the base of each column indicate rats/group.

Figure 4. Re-exposure to amphetamine-paired contextual stimuli rapidly increases spine tip diameter and spine density in c-Fos+ but not c-Fos- neurons in the NAcc shell.

One week following exposure, rats were placed in the open fields for the 30-minute conditioning test. Brains were harvested immediately after the test. (**A**) The number of medium-sized (0.45-0.60 μm) spine tip diameters was significantly increased in the c-Fos+ neurons of Paired relative to Unpaired and Control rats. (**B**) This was not observed in c-Fos- neurons. Similarly, a significant increase in spine and spine tip density was observed in the c-Fos+ but not c-Fos- neurons of Paired relative to Unpaired and Control rats (**C**). Together, these findings indicate a rapid amphetamine-paired stimulus-induced increase in de novo spines with spine tip characteristics capable of enabling enhanced transmission. (**D**) DiI labeled dendritic segment following deconvolution processing. Images were taken on a Leica SP5 confocal microscope (63X oil with 4X digital zoom). (**E**) Reconstruction of the dendritic segment using the Imaris software filament tracer module. (**F**) Surface reconstruction of the dendritic segment using Imaris. *****, $p<0.05$; ******, $p<0.01$; *******, $p<0.001$; relative to Unpaired and Control. Numbers in parentheses indicate rats, cells/group. Measures obtained from the cells of each rat (2-6/rat) were averaged and ANOVA conducted on these values. ANOVA conducted on un-averaged cell values yielded identical results, indicating homogeneity between cells for the different group measures (see Supplement 7).